Cytotoxic isoferulic acidamide from *Myricaria germanica* (Tamaricaceae)

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Tamgermanitin, a unique *N-trans*-Isoferuloyltyramine, together with the hitherto unknown polyphenolics, 2,4-di-*O*-galloyl-(α/β)-glucopyranose and kaempferide 3,7-disulphate have been isolated from the leaf aqueous ethanol extract of the false tamarisk, *Myricaria germanica* DESV. In addition, 18 known phenolics were also separated and characterized. All structures were elucidated on the basis of detailed analysis of 1D- ^{1}H and ^{13}C NMR, COSY, HSQC, HMBC and HRFTESIMS spectral data. The extract, its chromatographic column fractions and the isolated isoferuloyltyramine, tamgermanetin demonstrated potential cytotoxic effect against three different tumor cell lines, namely liver (Huh-7), breast (MCF-7) and prostate (PC-3). The IC_{50}"s were found to be substantially low with low-resistance possibility. DNA flow-cytometric analysis indicated that column fractions and tamgermanetin enhanced pre-G apoptotic fraction. Both materials showed inhibiting activity against PARP enzyme activity. In conclusion, we report the isolation and identification of a novel compound, tamgermanitin, from the aqueous ethanol extract of *Myricaria germanica* leaves. Further, different fractions of the extract and tamgermanitin exhibit potent cytotoxic activities which warrant further investigations.

Introduction

Natural products, including plant phenolics, provide a major source of chemical diversity that has consistently proven its value for the development of novel drugs for more effective antineoplastic agents. Nature provides candidate compounds which have more "drug-like" properties (i.e., in terms of absorption and metabolism) as well as a greater chemical diversity (i.e., to allow for structure-activity studies).[1] In an in vitro survey of preventive agents against tumor promotion from medicinal plants, polyphenols such as (-)-epigallocatechin gallate (EGCG), pentagalloyl-glucose,[2] pedunculagin and chebulinic acid, etc. were found to possess promising anticancer activity, e.g., they exhibit a competitive binding activity to TPA receptor in a particulate fraction of mouse skin.[3] On the basis of the above given criteria, we will investigate in the present study the cytotoxicity and the constitutive phenolics of *Myricaria germanica* DESV, aiming to achieve candidate phenolics which could be used for the development of effective antineoplastic agents.

The genus *Myricaria* belongs to the family Tamaricaceae, which comprises four genera and about 110 species widely distributed in Europe, Africa and Asia.[4] Many of these species grow on saline soils, tolerating up to 15,000 ppm soluble and can also tolerate alkaline conditions. In view of this fact, the capability of these plants on synthesizing and accumulating sulfate conjugates of flavonols, phenyl propanoids and other phenolics[5,6] is thus not all that surprising. Among the 10 *Myricaria* species, *Myricaria germanica* DESV, known in English as German false tamarisk or German tamarisk, is growing in temperate regions especially in the Mediterranean area. It is nearly allied to *Tamarix* plants, but it differs in having 10 stamens to each flower. The branches are erect, rather sturdier than in the true *Tamarix*, the leaves are pale glaucous hue and the flowers are white or rosy in June.[7] The species is a folk medicinal plant whose bark extract has been used in folk medicine for jaundice, while the infusion of the leaves was used as analgesic and was found to possess antimicrobial activity and to control chronic bronchitis.[8,9] The only previous phytochemical investigation of the plant leaf cuticular waxes has led to the isolation and characterization of a number of long-chain alkanediols.[10] Phytochemical investigation of its constitutive phenolics has not been adequately covered. We have previously explored the chemistry and biology of the phenolic constituents in Tamaricaceae.[11-14] In the present study we describe the isolation and structure determination of 20 phenolics (1–20) of *Myricaria*

germanica, including three hitherto unknown natural products, namely, *N-trans*-Isoferuloyltyramine, *N-trans*-3-hydroxy 4-methoxy cinnamoyltyramine (20), which we named tamgermanitin and 2,4-di-*O*-galloyl-(α/β)-glucopyranose (12) together with kaempferide 3,7-di-sodium sulfate (2). Tamgermanitin (20) is of special interest as it represents the first reported natural occurrence of an isoferulic acid amide. The analogs amide of the positional isomer, ferulic acid has been characterized before, from *Achyranthes bidentata*[15] and *Solanum tuberosum*.[16]

Results and Discussion

Isolation and identification. Following column chromatographic fractionation of the extract obtained by extraction of the leaves of *M. germanica* by aqueous ethanol, 20 compounds (1–20) were isolated. Conventional and spectral analysis mainly by NMR spectroscopy and by mass spectrometry indicated that three of these compounds (2, 12, 20) have not been reported before to occur in nature.

Compound 2 (**Fig. 4**) was isolated as an off-white amorphous powder, which exhibited chromatographic and anionic character on electrophoretic analysis similar to those of anionic flavonols.[17] UV absorption maxima in MeOH and after addition of diagnostic shift reagents[18,19] showed no shift with NaOAc or with $NaOAc/H_3BO_3$, a small shift with NaOMe and 28 nm shift with $AlCl_3$+HCl. These data were consistent with 3,7,4'-trisubstituted kaempferol structure. On mild acid hydrolysis (0.1 N aq. HCl at 100 C for 3 min) (2) yielded two intermediates (2a) (major, yellow spot on PC under UV light) and (2b) (minor, dark purple spot on PC under UV light). The aqueous acidic hydrolysate gives a white ppt. with aq. $BaCl_2$ to prove the presence of SO_4 group. Atomic absorption analysis confirmed that the SO_4 radical(s) exists in the molecule of (2) as sodium sulfate. Intermediates (2a and 2b) were individually separated by preparative paper chromatography. Their chromatographic, electrophoretic properties, UV absorption and ¹H NMR spectral data proved a 7,4''-disubstituted kaempferol structure for 2a and a kaempferol 3,4''- disubstituted structure for (2b). Complete hydrolysis of the parent Compound 2 (0.1 N aq. HCl at 100°C for 15 min) yielded kaempferol 4''-methyl ether, kaempferide (CoPC, UV, EIMS, ¹H and ¹³C NMR) and sodium sulfate ($BaCl_2$ test and atomic absorption analysis), a result which, when incorporated with the above given analytical data, proved the identity of (2a) as kaempferide 7-OSO_3Na and that of (2b) as kaempferide 3-OSO_3Na. Consequently, Compound 2 is suggested to be kaempferide 3,7-di-sodium sulfate. ESI-FTMS (negative ions) of 2: m/z = m/2 = 229.19110 = (M-2Na)²⁻, calc: 229.18502 corresponding to a molecular formula of $C_{16}H_{10}O_{12}S_2$. The spectrum exhibited also ions at *m/z* 480 (M - Na - H)⁻, 423 (M - SO₃ - H)⁻ and 343 (M - 2SO₃ - H)⁻, corresponding to a molecular weight 504. This and the above given data proved that (2) is kaempferide 3,7-di-sodium sulfate. Further support for this view was obtained through NMR spectral analysis. The ¹H spectrum of (2) revealed in the aromatic region a pattern of signals, though similar to that of the aglycone, keampferide (see Experimental), yet a distinction could be made through the recognition of the downfield shift of the proton signals of H - 6 and H - 8 (δ ppm 6.45 and 6.81, respectively), in comparison with the signal at δ ppm 6.20 and 6.45 of the corresponding protons in the spectrum of the free aglycone. This is obviously due to sulphation at position 7 of the kaempferide moiety. From the ¹³C spectrum of (2) the recognized up field shift (Δ δ = 3.1 ppm) of the resonance of C-3 and the accompanying downfield shift (Δ δ = 9.8 ppm and Δ δ = 2.3 ppm) of the signals of carbons C-2 and C4, respectively, all in comparison with the chemical shift of the corresponding signal in the spectrum of the aglycone (see Experimental) are attributed to sulphation at C-3 of the aglycone moiety. Similar set of shifts was recognized due to sulphation at C-7. Such shifts are well known.[17,20] Other resonances in this spectrum exhibited chemical shift values which were in close agreement to the achieved structure of compound (2) as kaempferide 3,7-di-sodium sulfate, a natural product, which represents, to the best of our knowledge, a new natural product.

Compound 12 (**Fig. 5**) was obtained as an amorphous powder which possesses galloyl ester-like characters (intense blue color with $FeCl_3$, rosy red color with KIO_3[21] and UV spectral maximum in MeOH at 274 nm). ESIMS analysis (negative mode) established that Compound 12 was a digalloyl glucose with a M_r of 484 [m/z = 483, (M - H)⁻] as was confirmed by HRESIMS, *m/z*: 483.3563 (calc.: 483.3572) for molecular formula $C_{20}H_{20}O_{14}$ On normal acid hydrolysis (2N aqueous HCl at 100°C for 3 h) (12) yielded gallic acid (CoPC, UV, ¹H and ¹³CNMR spectral analyses) together with glucose (CoPC), while on controlled acid hydrolysis (0.5 N aq. HCl, 100°C, 3 h) it gave, beside glucose and gallic acid (CoPC), an intermediate (12a), which was extracted from the hydolysate by EtOAc and purified by preparative paper chromatography. This was shown to have a M_r of 332 [negative ESIMS: (M - H)⁻, m/z 331] and UV spectral maximum in MeOH at 273 nm, thus suggesting its structure to be a monogalloyl glucose. To determine the site of attachment of the two galloyl moieties to the glucose core in the molecule of (12), ¹H NMR spectral analysis was then performed. The spectrum, recorded in DMSO-d_6, revealed, in addition to the characteristic singlets of the galloyl moieties at 6.93, 6.94, 6.95 and 6.97, two different patterns of proton signals belonging to an α/β anomeric mixture of disubstituted glucose, whereby a pair of doublets, centered at δ 5.19 (*J* = 3.5 Hz) and at 4.70 (*J* = 8 Hz), were recognized and assigned to the α- and β-anomeric glucose protons, respectively, thus indicating a free anomeric OH group. The spectrum also showed two downfield glucose proton resonances at 4.62 (dd, *J* = 3.5 Hz and *J* = 8 Hz) and at 4.72 (*t*, *J* = 8 Hz), assignable to the H-2 glucose protons in both α- and β-anomers, respectively. The downfield location of both resonances is clearly due to galloylation of their geminal OH groups. This assignment was based on the observation that the double-doublet mode of splitting of the signal located at 4.62 is typical of an axial H-2 proton in α-$^4C_1$-glucose core, being coupled to both the α-anomeric equatorial proton (*J* = 3.5 Hz) and to the axial H-3 proton (*J* = 8 Hz) of the same moiety. Galloylation at 4-position of the glucose core was evidenced by the two low-field proton signals located at δ 4.83 (*t*, *J* = 8 Hz) and 4.72 (*t*, *J* = 8 Hz), assignable to H-4α and H-4β, respectively, an assignment which was confirmed by

measurement of a ^{1}H-^{1}H-COSY spectrum. In addition, the values of the above coupling constants indicated that the α- and β-glucose cores of 12 are adopting a $^4C_1$ conformer. The weight of evidence given above, proved that compound 12 is 2,4-di-O-galloyl-(α/β)-$^4C_1$-glucose. Final proof of structure was then achieved through ^{13}C NMR spectral analysis which afforded a spectrum containing essentially double signals for most of the glucose and galloyl carbons. Resonances were assigned by comparison with the ^{13}C NMR data, reported for similar galloyl glucoses,[11,14] as well as by consideration of the known α- and β-effect caused by esterifying the sugar hydroxl groups.[14] In the received spectrum, the α- and β-anomeric carbon signals were readily identified from their characteristic chemical shift values (δ ppm 89.7, C-1α and 94.8, C-1β). Attachment of one of the galloyl moiety to C-2 of the glucose core followed from the β-upfield shift recognized for the resonances of both the vicinal C-1 and C-3 carbons (β-effect) and from the downfield shift of the resonances of the C-2 carbon (α-effect). Attachment of the second galloyl moiety to C-4 of glucose was evidenced by the β-upfield shift recognized for the vicinal carbon (C-3 and C-5) resonances (all in comparison with the chemical shifts of the corresponding carbon resonances in the spectrum of unsubstituted α/β glucopyranos). In both anomers, the resonances of C-2 were found to be shifted downfield (α-effect) at δ 75.8 (C-2-α) and 76.7 (C-2-β), while those of C-4 were shifted downfield to 71.6 (C-4-α) and 73.8 (C-4-β). Other resonances in this spectrum exhibited chemical shift values which were in accordance with the proposed structure. All assignments were confirmed by HSQC and HMBC experiments. Furthermore, the measured chemical shift values of the glucose carbon resonances proved that this moiety existed in the pyranose form, thus confirming the final structure of 12 to be 2,4-di-O-galloyl-(α/β)-$^4C_1$-glucopyranose, a secondary metabolite, which has not been reported before in literature (Compound 12).

Compound (20), isolated as a pale yellow oil, which gave a positive reaction with $FeCl_3$ reagents. Spots of (20) on Paper Chromatogram appeared with mauve color, which turned yellow when fumed with ammonia. It was analyzed for the molecular formula $C_{18}H_{19}O_4N$ on the basis of ^{1}H, ^{13}C NMR and HRESIMS [(M - H)$^-$: 312.1203, calc.: 312.3495]. IR absorbance bands recorded for 20 at v_{max} (KBr) cm^{-1}: 1204, 1430 (C-N stretching), 1625 (- C = O), 2921, 3015 (N-H stretching), 3120 and 3625 cm^{-1}, thus proving the presence of hydroxyl and amide carboxyl. The compound presented UV absorption in MeOH at $λ_{max}$ 295 and 315 nm, which were reminiscent of a phenyl propanoid system. Compound 20 yielded isoferulic acid (mauve color spot on PC, which turned yellow when fumed with ammonia, CoPC, ^{1}H and ^{13}C NMR) and tyramine hydrochloride (EI-MS, UV absorption and ^{1}H NMR) on acid hydrolysis [2 N aqueous/methanolic (1:1), HCl, 3 h, 100°C]. ^{1}H NMR spectrum of 20 displayed a pair of doublets, each of J = 16 Hz, at δ ppm 6.26 and 7.52 ppm attributable to trans-olefinic protons and a distinct set of aromatic protons together with a methoxyl signals assignable to the 3-hydroxy-4-methoxyphenyl moiety of isoferulic acid [δ ppm 7.06 (d, 1H, J = 2 Hz, H-2); 7.05 (dd, 1H, J = 2 Hz and J = 8 Hz, H-6); 6.92 (d, 1H, J = 8 Hz, H-5); 3.87 (s, 3H, OMe-3)]. The spectrum also exhibited a second distinct set consisting of a pair of aromatic proton resonances, each integrated to two equivalent protons, and two sp^3 methylenic signals all belonging to a phenethyl moiety at δ 6.68 (d, 2H, J = 8 Hz, H-3' and H-5'); 7.04 (d, 2H, J = 8 Hz, H-2' and H-6'); 3.48 (m, 2H, H-8'); 2.54 (t, 2H, J = 7.3 Hz H-7'). The ^{13}C spectrum of 20 contained 18 lines, and the DEPT spectrum established the presence of one carbonyl, three quaternary sp^2, nine protonated sp^2 carbons and three oxygenated sp^2 carbons together with two sp^3 methylenic carbons and one sp^3 oxygenated methyl carbon. Direct correlation observed in the ^{1}H-^{1}H COSY, HSQC and HMBC spectra of 20 allowed unambiguous assignment of protons, protonated and quaternary carbons. Analysis of ^{1}H-^{1}H COSY NMR spectroscopic data allowed -CH_2-CH_2- and CH = CH-subunits to be defined. The connectivity between the protons of these subunits with carbons in the 4-hydroxyphenyl and the carbons of the 3-hydroxy 4-methoxy phenyl moieties was demonstrated by interpretation of the HMBC correlation data. The observed ^3J correlations in this spectrum showed that proton H-8' (see Formula) (δ 3.48) correlates to the carbonyl carbon C-9 (δ 165.9) and to the quaternary p-hydroxyphenyl C-1' carbon (δ 129.95). Among the ^3J correlations recognized one was found correlating the methoxyl proton signal at (δ 3.87) to the aromatic carbon C-4 at (δ 150.7), another correlated the olefinic proton H-7 at (δ 7.52) to carbons C-2 at (δ 113.26), C-6 at (121.57) and to the carbonyl carbon C-9 at 165.90 and a third correlated the sp^3 methylenic protons 2H-8' at (δ 3.48) to the carbonyl carbon C-9 at (δ165.90) and to the phenethyl carbons (C-1') at δ 129.95. The recognizable ^2J correlations recorded in this spectrum (see Experimental) were in accordance with the achieved structure. These and the above given data finally confirmed the structure of Compound 20 to be N-trans-3-hydroxy 4-methoxy cinnamoyl-tyramine, for which we give the name tamgermanetin, a unique isoferuloyl derivative, Tamgermanetin (**Fig. 6**).

In addition, the known compounds, 3-methoxygallic 5-OSO_3Na (1), kaempheride 3-OSO_3Na (3), tamarexitin 3-OSO_3Na (4), gallic acid (5), 3-methoxygallic acid (6), 2,3-di-O- galloyl-(α/β)-glucose (7), quercetin 3-O-β-glucuronide (8), kaempferol 3-O-β-glucuronide (9), tamarixetin 3-O-β-glucuronide (10), 1,3-di-O-galloyl-β-glucose (11), 2,6-di-O- galloyl-(α/β)-glucose (13), tamarixellagic acid (14), kaempferol 3-O-α-rhamnopyranoside (15), quercein 3-O-α-rhamnopyranoside (16), kaempferide (17), tamarixetin (18) and quercetin (19), were also isolated from the investigated extract and were identified by applying the conventional and spectral methods of analysis.

Besides, the analytical data received during the course of the present study proved that the phenolic profile of *Myricaria germanica* is similar to those reported for Tamaricaceous plants.[11-14]

Biological assays. *Cytotoxicity assessment.* SRB-U assay[22] was used to assess the cytotoxicity of the crude extract and its column fractions against three different tumor cell lines over concentration range 0.01–100 µg/ml. Doxorubicin was used as a positive control. The crude extract per se showed considerable potency against PC-3, Huh-7 and MCF-7 cell lines with IC_{50} values of 6.5, 2.85 and 0.2 µg/ml, respectively. MCF-7 cell line

Table 1. Cytotoxicity parameters of the crude extract and its isolated fractions against different tumor cell lines

Fraction	Identified compounds	PC-3		Huh-7		MCF-7	
		IC$_{50}$ (μg/ml)	R- Fr. (%)	IC$_{50}$ (μg/ml)	R- Fr. (%)	IC$_{50}$ (μg/ml)	R- Fr. (%)
Whole extract		6.5	0.0	2.85	0.55	0.2	8.4
I	3-Methoxygallic 5-OSO$_3$Na **1**	6.2	N/A	11.5	18.2	2.02	0.0
II	Kaempferide 3,7-disodium sulfate **2**	1.5	6.5	0.84	0.0	1.2	0.0
III	Kaempheride 3-OSO$_3$Na **3**, Tamarexitin 3-OSO$_3$Na **4**	2.7	5.7	1.8	0.0	0.2	13.5
IV	Gallic acid **5**, 3-Methoxygallic acid **6**	1.4	8.1	0.03	3.7	0.13	0.0
V	2,3-di-*O*- Galloyl-(α/β)-glucose **7**	0.3	7.1	0.33	6.1	1.1	0.0
VI	Quercetin 3-*O*-β-glucuronide **8**, kaempferol 3-*O*-β-glucuronide **9** Tamarixetin 3-*O*-β-glucuronide **10**	0.22	1.4	0.22	8.0	0.25	0.0
VII	1,3-di-*O*-Galloyl-β-glucose **11**, 2,4-di-*O*-(α/β) Galloyl glucopyranose **12**	0.61	6.7	0.75	0.0	0.56	0.0
VIII	2,6-di-*O*- Galloyl-(α/β)-glucose **13**	0.4	0.3	0.13	10.2	0.15	0.0
IX	Tamarixellagic acid **14**	0.13	0.0	0.03	5.9	0.16	0.0
X	kaempferol 3-*O*-α-rhamnopyranoside **15**, Quercetin 3-*O*-α-rhamnopyranoside **16**	1.3	2.4	0.9	4.7	1.9	0.5
XI	Kaempferide **17**, Tamarixetin **18**, Quercetin **19**	2.61	0.0	0.65	0.0	0.33	5.7
XII	Tamgermanitin **20**	0.65	5.1	0.3	4.1	1.02	0.0
Positive control	Doxorubicin	0.63	5.4	1.5	0.0	0.13	0.0

showed relatively high resistance fraction after treatment with the crude extract with R-fraction of 8.4% while there were negligible R-values for PC-3 and Huh-7 cells (0 and 0.55%, respectively). Column fraction IX (tamarixellagic acid) showed the most potent cytotoxicity against PC-3 prostate cancer cell line (IC$_{50}$ = 0.13 μg/ml) with 0.0% R-fraction; the other column fractions showed less potent cytotoxic effects with IC$_{50}$'s ranging from 0.22 to 6.2 μg/ml. In Huh 7 liver cancer cell line. Column fractions IV and IX (tamarixellagic acid) showed the most potent cytotoxic profile with IC$_{50}$ of 0.03 μg/ml for both fractions with R-value of 3.7% and 5.9%, respectively. Other column fractions showed much lower but considerable cytotoxic profile against Huh-7 cell line with IC$_{50}$ values ranging from 0.13–11.5 μg/ml. In case of MCF-7 breast cancer cell line, column fractions IV, VIII and IX (tamarixellagic acid) showed the highest cytotoxic profile with IC$_{50}$'s of 0.13 μg/ml, 0.15 μg/ml and 0.16 μg/ml, respectively and the R-fraction was 0.0%. The other fractions showed milder but considerable cytotoxic effect with IC$_{50}$'s ranging from 0.2–2.02 μg/ml (**Table 1**). Collectively, if we compare the obtained data with those of doxorubicin, it should be mentioned that tamarixellagic acid and tamgermanetin showed promising cytotoxic profiles with potent IC$_{50}$'s and R-values against all the cell lines tested herein.

Assessment of cell cycle distribution. DNA flow-cytometry was used to assess the effect of tamarixellagic acid and tamgermanetin on the cell cycle distribution of Huh-7 and MCF-7 cell lines after treatment for 24 h. In Huh-7, tamarixellagic acid and tamgermanetin significantly decreased the non-proliferating cell fraction (G$_0$/G$_1$-phase) from 65% (**Fig. 1A**) to 57% (**Fig. 1B**)

and 48% (**Fig. 1C**), respectively. Treatment with tamarixellagic acid induced minimal compensatory increase in S-phase while tamgermanetin showed mild increase in G$_2$/M-phase and strong increase in the pre-G apoptotic fraction (**Fig. 1D**). With respect to MCF-7 cell line, both tamarixellagic acid (**Fig. 2B**) and tamgermanetin (**Fig. 2C**) significantly increased the pre-G apoptotic fraction compared with control (**Fig. 2A**) from 5.3–10.5% and 8.8%, respectively. Tamarixellagic acid significantly decreased the S-phase with recorded increase in response to treatment with tamgermanetin. On the other hand, IX increased G$_2$/M fraction (10.6%) while tamgermanetin depleted the mitotic cells to 2.1% compared with control cells (7.8%).

Assessment of PARP and caspase-3 enzyme activity. PARP is a family of proteins involved in a number of cellular processes involving mainly DNA repair and programmed cell death and hence negatively influences apoptosis pathway after cytotoxic effects. Exposure of cell-free PARP enzyme to the pre-determined IC$_{50}$''s of tamarixellagic acid and tamgermanetin abolished the enzyme activity by 63.4 and 67.9%, respectively. The enzyme inhibition was validated by incubating the enzyme with the IC$_{50}$ of positive control PARP inhibitor (3-amino benzamide-3AB), which inhibited the enzyme by 52.1% (**Fig. 3A**). In addition to sensitizing effect of tamarixellagic acid and tamgermanetin to DNA damage, the effect on the activity of caspase-3 was assessed in Huh-7 cell line. Tamarixellagic acid and tamgermanetin increased the activity of caspase-3 activity by 154.5 and 175% respectively (**Fig. 3B**). Accordingly, tamarixellagic acid and tamgermanetin per se induce tumor cells to proceed via apoptotic pathway in addition to sensitizing tumor cells to DNA damaging agents.
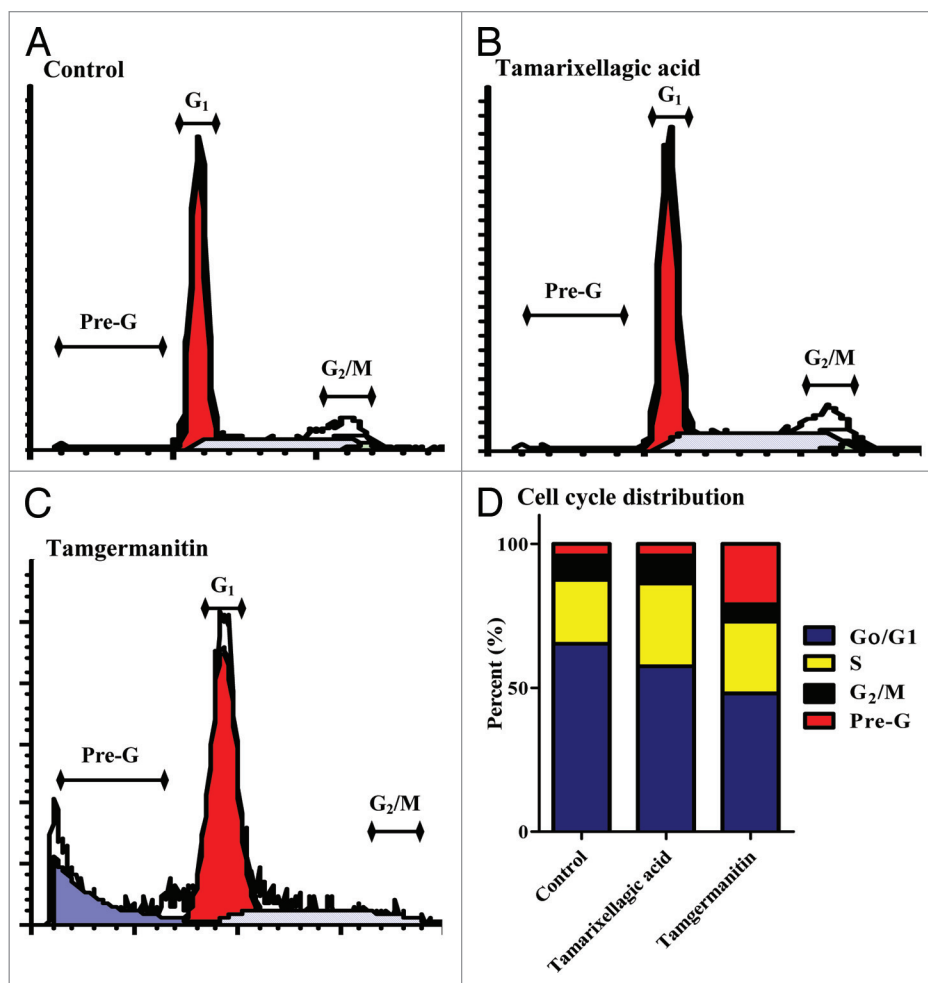
Figure 1. Effect of tamarixellagic acid and tamgermanitin on the cell cycle distribution of Huh-7 cells. Cells were exposed to tamarixellagic acid (**B**) and tamgermanitin (**C**) for 24 h and compared with control cells (**A**). Cell cycle distribution was determined using DNA cytometry analysis and different cell phases were plotted (**D**) as percent of total events (n = 3).

escape apoptosis has been highlighted and inhibitors of PARP enzyme sensitized several tumor types to the effect of anticancer drugs.[23,24] Inhibition of PARP enzyme activity by tamarixellagic acid and tamgermanitin might, at least partly, sensitize tumor cells to death signal. This assumption is supported by the low R-fraction in all tested cell lines treated with tamarixellagic acid and tamgermanitin. Besides, tamarixellagic acid and tamgermanitin per se induced death signal as evidenced by the significant increase in the pre-G apoptotic cell fraction and the elevated caspase-3 activity in Huh-7 cell line. It is noteworthy that both materials increased the accumulation of cells at G_2/M phase. This suggests that tamgermanitin-induced apoptosis might involve interaction with microtubules. This suggestion gains support by the recorded ability of amide phenolic compounds like capsaicin and phenolic compounds like the resveratrol derivative, 2, 3", 4, 4", 5"-pentamethoxy-trans-stilbene, to interfere with microtubule function. In conclusion, we report the isolation and identification of a novel compound, tamgermanitin, from the aqueous ethanol extract of *Myricaria germanica* leaves. Further, different fractions of the extract and tamgermanitin exhibit potent cytotoxic activities which warrant further investigations.

Materials and Methods

General experimental procedures. ^{1}H NMR spectra were measured by a Jeol ECA 500 MHz NMR spectrometer. [1]H chemical shifts (δ) were measured in ppm, relative to TMS and ^{13}C NMR chemical shifts to DMSO-d_6 or $(CD_3)_2CO$ and converted to TMS scale by adding 39.5 or 30.0, respectively, as stated in each case. Typical conditions: spectral width = 8 kHz for 1 H and 30 kHz for 13 C, 64 K data points and a flip angle of 45°. FTMS spectra were measured on a Finnigan LTQ-FTMS (Thermo Electron) (Department of Chemistry, Humboldt-Universität zu Berlin). UV recording were made on a Shimadzu UV–Visible-1601 spectrophotometer. Flame atomic absorption analysis was performed on a Varian Spectra-AA220 instrument, lamp current: 5 ma, fuel: acetylene, oxidant: air, slit width: 0.5 nm. $(\alpha)_{25}^D$ were measured on a Kruess polarimeter-8001 (A. Kruess. Optronic). Paper chromatographic analysis was performed on Whatman No. 1 paper, using solvent systems: (1) H_2O; (2) 6% HOAc; (3) BAW, (*n*- BuOH-HOAc-H_2O, 4:1:5, upper layer).

We present herein, a unique natural phenolic amide, *N*-isoferuloyltyramine with promising cytotoxic effect against three different types of solid tumors, namely, breast, prostate and liver cancers. The promising cytotoxicity of the crude extract of *Myricaria germanica* mandated further fractionation whereby, column fractions VI and IX have shown the most promising cytotoxic profile in terms of IC_{50} and R-fraction. Three flavonol glucuronoids, quercetin 3-*O*-β-glucuronide, kaempferol 3-*O*-β-glucuronide and tamarixetin 3-*O*-β-glucuronide, have been identified as the major constituents of VI. This finding might explain the superior cytotoxicity of that column fraction. The remarkable activity of tamarixellagic acid could be attributed to the typical ellagitannin monomer. More interesting is the distinguished activity determined for the unique acidamide, *N*-isoferuloyltyramine, tamgermanitin. To further substantiate the observed cytotoxicity, the potential effects of tamarixellagic acid and tamgermanitin against PARP enzyme activity was examined. Both materials strongly inhibited the PARP enzyme activity. Distinguished role of PARP enzyme in DNA-repair and
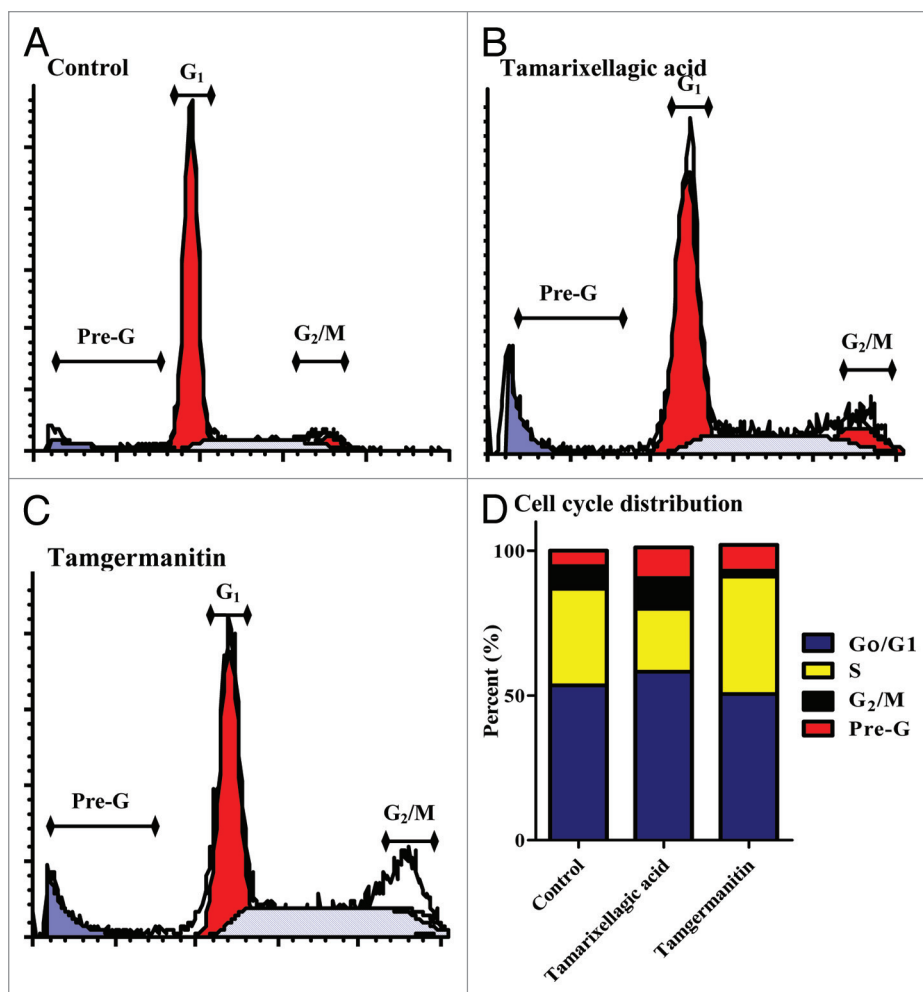
Figure 2. Effect of tamarixellagic acid and tamgermanitin on the cell cycle distribution of MCF-7 cells. Cells were exposed to tamarixellagic acid (**B**) and tamgermanitin (**C**) for 24 h and compared with control cells (**A**). Cell cycle distribution was determined using DNA cytometry analysis and different cell phases were plotted (**D**) as percent of total events (n = 3).
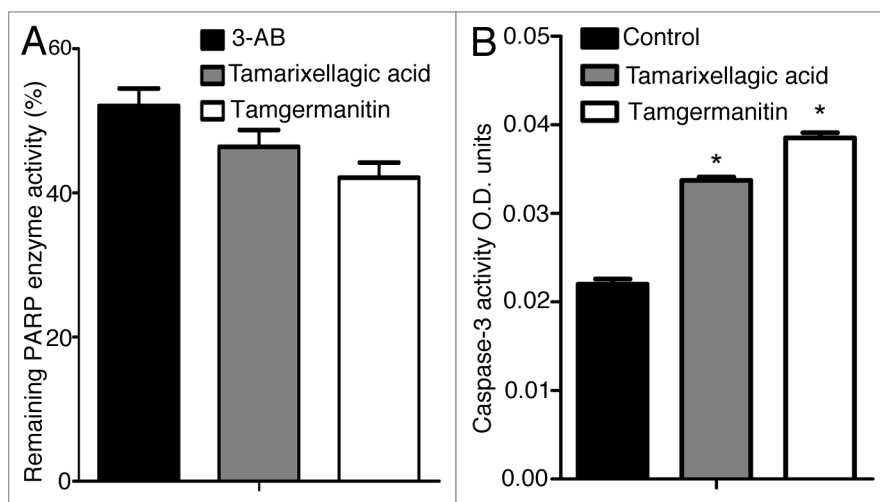

Figure 3. Effect of tamarixellagic acid and tamgermanitin on PARP (A) and caspase-3 (B) enzyme activity was assessed in cell-free system and in Huh-7 cells, respectively. Data are presented as mean ± SEM; (n = 3).

Plant materials. Collection of the leaves of *Myricaria germanica* was made at the Botanical garden of the University Bonn, in June 2009. Authentication was performed by Dr. Peter König, Botanical garden, Ernst-Moritz-Arndt-University Greifswald. Voucher specimens were deposited at the herbarium of the NRC.

Extraction and isolation. Fresh *M. germanica* leaves (200 g) were homogenized in EtOH–H_2O (3:1) mixture (three extractions each with 250 ml). A portion, 35 g of the dried filtrate (41 g) of the homogenate was applied to a Sephadex LH-20 (250 g) column (100 × 2.5 cm) and eluted with H_2O followed by H_2O/MeOH mixtures of decreasing polarities to yield 12 fractions (I–XII). The received fractions were individually subjected to 2DPC. Compound 1 (71 mg) was isolated pure from fraction I (2.3 g, eluted with H_2O) by repeated (thrice) precipitation with acetone from a concentrated aqueous solution of this fraction (903 mg). Compound 2 (85 mg) was obtained pure by re-fractionation of 1.2 g of fraction II (1.6 g, eluted with H_2O) over Sephadex LH-20 (17 g) column (30 × 2 cm) and elution with water. Compounds 3 and 4 were individually isolated pure (112 mg and 96 mg, respectively) through repeated preparative PC of the material of fraction III (661 mg, eluted with 10% MeOH), using water as solvent. Each of compounds 5 and 6 were separated pure (41 and 43 mg, respectively) from 240 mg of fraction IV (940 mg, eluted with 20% MeOH) by applying Sephadex LH-20 column(12 g, 30 × 2 cm) fractionation and elution with *n*-BuOH water saturated. Polyamide column (25 g, 45 x 2.5 cm) fractionation of 880 mg amount of fraction V (1.9 g, eluted with 30% MeOH) and elution with 30% aqueous MeOH yielded pure samples of **7** (49 mg).

Compounds 8, 9 and 10 were individually isolated pure (58, 40 and 29 mg, respectively) from fraction VI (3.2 g, eluted with 40% MeOH) by repeated Sephadex LH-20 (30 g) column (45 × 2.5 cm) fractionation of 2.6 g material of this fraction. Application of repeated prep. PC, using *n*-BuOH saturated with water as solvent on the material (1.1 g) of fraction VII (1.89 g, eluted with 50% MeOH) afforded pure samples

of compounds 11 (42 mg) and 12 (49 mg). Compound 13 was obtained pure (52 mg) by repeated precipitation (thrice) from a concentrated acetone solution of 302 mg of fraction VIII (1.58 g, eluted with 60% MeOH) by ether. Extraction of 406 mg material of fraction IX (2.5 g, eluted with 70% MeOH) with EtOAc, while hot, followed by filteration, concentration of the filterate, cooling to room temperature and addition of ether led to precipitation of compound 14, which was filtered off and re-precipitated (thrice) to give a pure sample (51 mg). Column (45 × 2.5 cm) fractionation of 1.19 g material of fraction X (1.90 g, eluted with 80% MeOH) over 35 g Sephadex LH-20 using n-BuOH saturated with H_2O for elution afforded pure sample of compounds 15 (48 mg) and 16 (37 mg). Compounds 17 (49 mg), 18 (38 mg) and 19 (19 mg) were individually isolated from 792 mg of the major column fraction XI (3.4 g, eluted by 90% MeOH) through repeated prep. PC using BAW as solvent. Compound 20 was separated pure as fraction XII (2DPC). Removal of the solvent under reduced pressure at 40°C afford an oil sample of 20 (112 mg).

Kaempferide 3,7-sodium disulphate (2). R_f-values: 0.85 (1), 0.73 (2), 0.25 (3). Electrophoretic mobility: 5.6 cm, on Whatman no. 3 MM paper, buffer solution of pH 2, H_2O-HCOOH-AcOH (89:8.5:2.5), 1 and 1/2 h, 50 v/cm. UV λ_{max} nm in MeOH: 265, 300 shoulder, 342; NaOMe: 270, 380; NaOAc: 264, 310, 342; NaOAc-H_3BO_3: 266, 300 shoulder, 340; $AlCl_3$: 270, 302, 345, 380 (shoulder); HCl (30 min): 270, 370. ESIMS (negative mode): m/z 480 $(M - Na - H)^-$, 423 $(M - SO_3 - H)^-$ and 343 $(M - 2SO_3 - H)^-$; HRESIMS, m/z: 480.3569 $(M - Na - H)^-$, (calc.: 480.3580). Mild Acid hydrolysis (30 mg in 10 ml aqueous methanol, 1:1, of 0.1 N aq. HCl at 100°C for 3 min) of 2: yielded 2a: [R_f-values: 0.45 (1), 0.40 (2), 0.26 (3); electrophoretic mobility: 2.5 cm. UV λ_{max} nm in MeOH: 265, 365; NaOMe: 263, 389; NaOAc: 264, 310, 364; NaOAc-H_3BO_3: 265, 300 shoulder, 360; $AlCl_3$: 270, 302, 345, 400 shoulder; HCl: 270, 368; ^{1}H NMR: δ ppm (500 MHz, DMSOd$_6$): 8.11 (2H, d, J = 8.5 Hz, H-2" and H-6"), 7.03 (2H, d, J = 8.5 Hz, H-3' and H-5'), 6.78 (IH, d, J = 2 Hz, H-8); 6.40 (1H, d, J = 2 Hz, H-6)] and 2b: [R_f-values: 0.48 (H_2O), 0.42 (HOAc), 0.30 (BAW); electrophoretic mobility: 3 cm. UV λ_{max} nm in MeOH: 267, 342; NaOMe: 270, 350 decomposition; NaOAc: 269, 310, 346; NaOAc-H_3BO_3: 267, 342; $AlCl_3$: 270, 304, 345, 400 shoulder; HCl: 270, 367; ^{1}H NMR: δ ppm: 8.10 (2H, d, J = 8.5 Hz, H-2" and H-6"), 7.15 (2H, d, J = 8.5 Hz, H-3' and H-5'), 6.43 (1H, d, J = 2 Hz, H-8), 6.21 (IH, d, J = 2 Hz, H-6)]. The hydrolysates gave white $BaCl_2$ precipitate; flame atomic absorption of the hydrolysates: sodium line at 589 nm. Complete acid hydrolysis of 2 (14 mg in 5 ml, 0.1 N aq. methanolic HCl, 1:1, at 100 C for 15 min.) yielded kaempferol 4"-methyl ether, kaempferide (separated and filtered off from the cooled hydrolysate): R_f-value: 0.92 (3); UV λ_{max} nm in MeOH: 267, 300 shoulder, 367; NaOMe: 280, 404; NaOAc: 272, 310, 384; NaOAc-H_3BO_3: 267, 300 shoulder, 364; $AlCl_3$: 270, 304, 345, 420 shoulder; 367 EIMS- m/z: 300 $[M]^+$; ^{1}H NMR of kaempferide: δ ppm 8.15 (2H, d, J = 8.5 Hz, H-2" and H-6"), 7.05 (2H, d, J = 8.5 Hz, H-3' and H-5'), 6.45 (IH, d, J = 2 Hz, H-8); 6.20 (1H, d, J = 2 Hz, H-6). ^{13}C of kaempferide: δ ppm 146.7 (C-2), 135.7 (C-3), 175.9 (C-4), 160.7 (C-5),
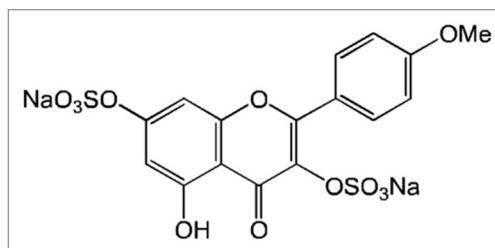

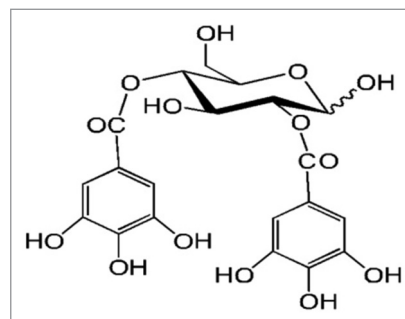**Figure 4.** Compound 2. Kaempferide 3,7-disodium sulfate.

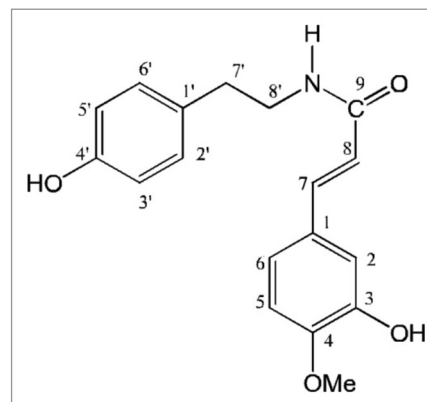**Figure 5.** Compound 12. 2,4-di-O-(α/β) galloyl glucopyranose.

Figure 6. Tamgermanetin. N-trans-3-hydroxy 4-methoxy cinnamoyl-tyramine.

98.2 (C-6), 163.9 (C-7), 93.5 (C-8), 156.2 (C-9), 103.0 (C-10), 123.2 (C-1'), 129.5 (C-2' and C-6'), 114.2 (C-3' and C-5', 160.2 (C-4'), 55.9 (C-4' OMe). ^{1}H NMR of 2: δ ppm 8.19 (2H, d, J = 8.5 Hz, H-2') and H-6', 7.1 (2H, d, J = 8.5 Hz, H-3' and H-5'), 6.82 (IH, d, J = 2 Hz, H-8); 6.45 (1H, d, J = 2 Hz, H-6). ^{13}C of 2: δ ppm 156.5 (C-2), 132.6 (C-3), 178.2 (C-4), 160.0 (C-5), 101.6 (C-6), 159.8 (C-7), 98.8 (C-8), 155.3 (C-9), 105.9 (C-10), 121.2 (C-1'), 129.8 (C-2' and C-6'), 114.4 (C-3' & C-5'), 160.6 (C-4'), 56.3 (C-4''' Me).

2,4-di-O-galloyl-(α/β)-$^4C_1$-glucopyranose (12). R_f values: 55 (l), 63 (2), 42 (3). $(\alpha)_D^{25}$ - 145° (c = 0.7, MeOH). UV data: λ_{max} (nm): 274. M_r 484, ESIMS (negative mode) m/z: 483 $(M - H)^-$, HRESIMS m/z: 483.3563 (calc.: 483.3572) for molecular

formula $C_{20}H_{20}O_{14}$. On complete acid hydrolysis (19 mg in 5 ml aq. two N HCl. 100" 3 h) compound 12 yielded glucose (CoPC) and gallic acid: R_f values: 35 (l), 63 (2), 72 (3); UV data: λ_{max} (nm): 272; EIMS: *m/z:* 272 (M)⁺; 1H NMR: δ ppm: 6.99 (*s*, H-2 and H-6); ¹³C NMR: δ ppm: 121.1 (C-1), 109.5 (C-2 and C-6), 145.3 (C-3 and C-5), 139.2 (C-4), 165.5 (C = O). On controlled acid hydrolysis (26 mg in 10 ml aq. 0.5 N HCl, l00°C 3 h) it yielded a monogalloyl glucose (l2a). Compound l2a: *R,-* values: 71 (I). 74 (2), 64 (3). UV data: λ_{max} (nm): 273. $M_r = 332$. ESIMS: *m/z*: 333 (M - H)⁻. ¹HNMR of 12: δ ppm: α-glucose moiety: 5.19 (*d, J = 3.5 Hz, H-l*), 4.62 (*dd, J = 8* and 3.5 Hz, H-2). 3.99 (t, *J =* 8 Hz, H-3). 4.83 (*t, J =* 8 Hz, H-4), 3.90 (*m*, H-5), 3.3–3.6 (m, H_2-6 protond overlapped with water signal); β-glucose moiety: 4.70 (*d, J = 8 Hz, H-1*), 4.72 (*t, J = 8 Hz, H-2*), 4.72 (*t, J = 8 Hz, H-4*), 3.76 (*m,* H-5), 3.3–3.6 (*m*, overlapped with water signal, H_2-6). ¹³C NMR of 12: α-glucose moiety: 89.7 (C-l), 75.8 (C-2), 72.4 (C-3), 71.4 (C-4), 70.3 (C-5), 61.0 (C-6); β-glucose moiety: 94.7 (C-l), 76.7 (C-2), 75.5 (C-3). 71.8 (C-4), 74.9 (C-5). 61.1 (C-6); galloyl moieties: 120.5, 119.9, 119.8 (C-l), 109.6, 109.4 (C-2 and C-6). 146.1, 146.0, 145.9 (C-3 and C-5). 139.1, 139.0, 138.8, 138.0 (C-4), 166.2, 165.9, 165.6, 165.1 (C = O).

N-trans-Isoferuloyltyramine, N-trans-3-hydroxy 4-methoxy cinnamoyltyramine, tamgermanitin (20). Pale yellow oil, mauve spot on PC under UV light, turning yellow on fuming with Ammonia. R_f-values: 0.10 (1), 0.19 (2), 094 (3); UV λ_{max} nm (MeOH): 220, 295, 315. IR ν cm⁻¹: 3625 (OH), 2921, 3015 (N-H stretching), 1590, 1625 (- C = O), 1430 (C-N stretching). ESI-MS, (negative mode), *m/z*: 312, (M - H)⁻; HRESI-MS: *m/z*: 312.1203, calc.: 312.3495 for $C_{18}H_{19}O_4N$. Normal acid hydrolysis (2 *N* aqueous/methanolic HCl, (1:1), 3 h, 100°C) followed by removal of MeOH under reduced pressure, yielded isoferulic acid, (extracted from the aq. hydrolysate by ether), R_f-values: 37 (1), 45 (2), 92 (3); UV λ_{max} (nm) MeOH: 240, 295, 325; ¹H NMR [$(CD_3)_2CO$, room temp.]: δ ppm: 7.51 (1H, *d, J =* 16 Hz, H-7), 7.13 (1H, *d, J =* 2 Hz, H – 2), 7.06 (1H, *dd, J =* 2 Hz and J = 8 Hz, H6), 6.93 (1 H, *d, J =* 8 Hz, H – 5), 6.26 (1H, *d, J =* 16 Hz, H – 8); ¨¹³C NMR: δ pap [$(CD_3)_2CO$, room temp.]:127.2 (C-1), 113.62 (C-2), 146.91 (C-3), 150.70 (C-4), 109.15 (5), 121.57 (C-6), 145.01 (C-7), 115.82 (C-8), 168.43 (C = O). The aq. hydrolysate, thus remained was treated with excess acetone and the formed precipitate was separated by filtration. It was identified to be tyramine hydrochloride, UV λ_{max} (nm) MeOH: 276, 282 (sh). EI MS, *m/z*: 137 (M)⁺, 107 (M-CH2NH2), 91, 78, 77, 44, 30. ¹H NMR (D_2O, Ref.: DSS), δppm: 2.9 (2H, *t, J =* 7 Hz), 3.2 (2H, *t, J =* 7 Hz), 6.85 (d, 2H, J = 8 Hz), 7.2 (d, 2H, J = 8 Hz). ¹H NMR of compound 20: ¹H NMR [$(CD_3)_2CO$, room temp.]: δ ppm: 7.52 (1H, *d, J =* 16 Hz, H-7), 7.07 (1H, *d, J =* 2.0 Hz, H-2), 7.05 (1H, *dd, J =* 8 Hz, and 2 Hz, H-6), 7.04 (2H, *d, J =* 8 Hz, H-2' and H-6'), 6.92 (1H, *d, J =* 8.0 Hz, H-5), 6.68 (2H, *d, J =* 8 Hz, H-3' and H-5'), 6.26 (1H, *d, J =* 16 Hz, H-8), 3.87 (3H, *s*, OMe-4), 3.48 (2 H, *m*, H-8'), 2.54 (2H, *t, J =* 7.7 Hz, H-7')]. ¹³C NMR and HMBC correlations: [$(CD_3)_2CO$, room temp.]: δ ppm: Isoferuloyl moiety: 129.96 (C-1), 113.62 (C-2, correlated in HMBC, to the olefinic proton H-7 at δ 7.52), 146.90 (C-3), 150.07 (C-4, correlated with the methoxyl proton signal at δ 3.87), 115.82 (5), 121.57 (C-6, correlated with the olefinic proton H-7 at δ 7.52), 139.41 (C-7),

115.84 (C-8), 165.90 (C-9, correlated with H-8' at δ 3.48; correlated with the olefinic proton H-7 at δ 7.52 and to the methylenic protons 2H-8' at δ 3.48), 55.44 (OMe-4); Tyramine moiety7: 129.95 (C-1', correlated in HMBC to H-8' at δ 3.48), 129.99 (C-2' and C-6'), 113.62 (C-3' and C-5'), 150.07 (C-4'), 34.77 (C-7'), 40.17 (C-8').

Biological methods. *Chemicals and drugs.* Sulfarhodamine was purchased from Sigma-Aldrich. Tricloroacetic acid and other materials were of the highest available commercial grade.

Cell culture. Human hepatocellular cancer cell line, Huh-7, breast cancer cell line, MCF-7 and prostate cancer cell line, PC-3, were obtained from the Egyptian National Cancer Institute. Cells were maintained in RPMI-1640 supplemented with 100 μg/ml streptomycin, 100 units/ml penicillin and 10% heat-inactivated fetal bovine serum in a humidified, 5% (v/v) CO_2 atmosphere at 37°C.

Cytotoxicity assays and viability analysis. The cytotoxicity of the crude extract and its column fractions were tested against Huh-7, MCF-7 and PC-3 cells by SRB assay as previously described (Skehan et al., 1990). Exponentially growing cells were collected using 0.25% Trypsin-EDTA and plated in 96-well plates at 1,000–2,000 cells/well. Cells were exposed to test compound for 72 h and subsequently fixed with TCA (10%) for 1 h at 4°C. After several washing, cells were exposed to 0.4% SRB solution for 10 min in dark place and subsequently washed with 1% glacial acetic acid. After drying overnight, TRIS-HCl was used to dissolve the SRB-stained cells and color intensity was measured at 540 nm. The dose-response curve of compounds was analyzed using E_{max} model (**Eq. 1**).

$$\% \text{ Cell viability} = (100 - R) \times \left(1 - \frac{[D]^m}{K_d{}^m + [D]^m}\right) + R$$

Where R is the residual unaffected fraction (the resistance fraction), D is the drug concentration used, K_d is the drug concentration that produces a 50% reduction of the maximum inhibition rate and m is a Hill-type coefficient. IC_{50} was defined as the drug concentration required to reduce fluorescence to 50% of that of the control (i.e., $K_d = IC_{50}$ when R = 0 and $E_{max} = 100$-R).

Analysis of cell cycle distribution. To assess the effect of the column fractions on cell cycle distribution, cells were treated with the pre-determined IC_{50} for 24 h and collected by trypsinization, washed with ice-cold PBS and re-suspended in 0.5 ml of PBS. Ten milliliter of 70% ice-cold ethanol was added gently while vortexing, and cells were kept at 4°C for 1 h and stored at -20°C until analysis. Upon analysis, fixed cells were washed and re-suspended in 1 ml of PBS containing 50 μg/ml RNase A and 10 μg/ml propidium iodide (PI). After 20 min incubation at 37°C, cells were analyzed for DNA contents by FACSVantageTM (Becton Dickinson Immunocytometry Systems). For each sample, 10,000 events were acquired. Cell cycle distribution was calculated using CELLQuest software (Becton Dickinson Immunocytometry Systems).

Determination of caspase-3 activity. To assess the effective phase of apoptosis, caspase-3 activity after treatment with different column fractions was assessed in Huh-7 cell line. Cells were harvested

after treatment for 24 h with the pre-determined IC_{50} of each fraction. Caspase-3 activity was determined using Quantikine® immunoassay kit (R&D Systems) according to the manufacturer's instructions. Activity of caspase-3 was normalized based on protein concentration in each sample.

Determination of Poly (ADP-ribose) polymerase (PARP) enzyme activity. To assess the effect of different column fractions on blocking DNA repair, PARP enzyme activity was assessed using cell-free system enzyme assay. The remaining PARP enzyme activity was determined after incubation with the pre-determined IC_{50} using PARP Universal Colorimetric Assay (R&D Systems) according to the manufacturer's instructions. Standard PARP enzyme inhibitor (3-amino-benzamide) was used against purified PARP enzyme to plot standard curve and the intensity of the color measured in samples are then read off the standard curve.

Statistical analysis. Data are presented as mean ± SD. Analysis of variance (ANOVA) with LSD post hoc test was used for testing the significance using SPSS® for windows, version 17.0.0. $p < 0.05$ was taken as a cut-off value for significance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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